IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	**Confirmation No. 9569**
Yoshio UMEZAWA et al.	:	Attorney Docket No. 2002_0426A
Serial No. 10/089,040	:	Group Art Unit 1652
Filed April 16, 2002	:	Examiner William A. Moore
PROBE FOR PROTEIN-PROTEIN INTERACTION ANALYSIS, AND METHOD OF USING IT FOR ANALYSIS OF PROTEIN-PROTEIN INTERACTION	:	Mail Stop: AMENDMENT

TRANSLATOR'S DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Boris Zhupanov, declare and say:

that I am thoroughly conversant in both the Japanese and English languages;

that I am presently engaged as a translator in these languages;

that the attached document represents a true English translation of the Japanese priority PCT application for the above-identified application entitled "Probe for Protein-Protein Interaction Analysis, and Method for Analysis of Protein-Protein Interactions Using Same ".

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 9th day of November, 2005.

B. Zhupanov

Translator

pGEX Vectors (GST Gene Fusion System)

Order Information

Product	Pack size	Info	Product Code	Price
Glutathione S-transferase Gene Fusion Vectors*				
pGEX-1λT *Eco*R I/BAP	5 µg		27-4805-01	country select
pGEX-2T	25 µg		27-4801-01	country select
pGEX-2TK	25 µg		27-4587-01	country select
pGEX-3X	25 µg		27-4803-01	country select
pGEX-4T-1	25 µg		27-4580-01	country select
pGEX-4T-2	25 µg		27-4581-01	country select
pGEX-4T-3	25 µg		27-4583-01	country select
pGEX-5X-1	25 µg		27-4584-01	country select
pGEX-5X-2	25 µg		27-4585-01	country select
pGEX-5X-3	25 µg		27-4586-01	country select
pGEX-6P-1	25 µg		27-4597-01	country select
pGEX-6P-2	25 µg		27-4598-01	country select
pGEX-6P-3	25 µg		27-4599-01	country select

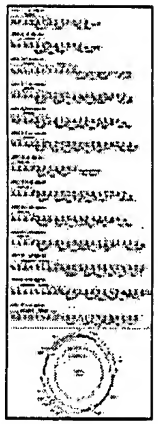
** All vectors include E. coli BL21 cells.*

Customers who bought these also bought:

Thrombin	500 units		27-0846-01	country select
PreScission Protease	500 units		27-0843-01	country select
GST Gene Fusion System Handbook	1		18-1157-58	country select
ALFEXPRESS SIZER 300	1 EA		27-4531-01	country select
Thermo Sequenase Cy5 Dye Terminator Kit	100 reactions		27-2682-01	country select
ALFEXPRES SIZER 50-500	1 EA		27-4539-01	country select

pGEX Vectors (GST Gene Fusion System)

Technical Information



Map of the glutathione S-transferase fusion vectors showing the reading frames and main features. Even though stop codons in all three frames are not depicted in this map, all thirteen vectors have stop codons in all three frames downstream from the multiple cloning site.

Download the pGEX sequence map in PDF format

All of the GST gene fusion vectors offer:

- **A tac promoter for chemically inducible, high-level expression.**
- An internal lac I^q gene for use in any E. coli host.
- Very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimizing effects on antigenicity and functional activity.
- PreScission™, thrombin, or factor Xa protease recognition sites for cleaving the desired protein from the fusion product.

Thirteen pGEX vectors are available (see figure). Nine of the vectors have an expanded multiple cloning site (MCS) that contains six restriction sites. The expanded MCS facilitates the unidirectional cloning of cDNA inserts obtained from libraries constructed using many available lambda vectors. pGEX-6P-1, pGEX-6P-2, and pGEX-6P-3 each encode the recognition sequence for site-specific cleavage by PreScission™ Protease, (see PreScission™ Protease) between the GST domain and the multiple cloning site. pGEX-4T-1, pGEX-4T-2, and pGEX-4T-3 are derived from pGEX-2T and contain a thrombin recognition site. pGEX-5X-1, pGEX-5X-2, and pGEX5X-3 are derivatives of pGEX-3X and possess a factor Xa recognition site.

pGEX-2TK is uniquely designed to allow the detection of expressed proteins by directly labeling the fusion products *in vitro* (1). This vector contains the recognition sequence for the catalytic subunit of cAMP-dependent protein kinase obtained from heart muscle. The protein kinase site is located between the GST domain and the MCS. Expressed proteins can be directly labeled using protein kinase and [γ-^{32}P]ATP and readily detected using standard radiometric or autoradiographic techniques. pGEX-2TK is a derivative of pGEX-2T; its fusion proteins can be cleaved with thrombin.

Cleavage of pGEX-6P GST fusion proteins occurs between the Gln and Gly residues of the recognition sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro [2]. Low temperature (5°C) digestion minimizes the degradation of the protein of interest. Because PreScission™ Protease has been engineered with a GST-tag, it can also be removed from the cleavage mixture simultaneously with the GST portion of the fusion protein. The pGEX-6P Expression Vectors permit convenient site-specific cleavage and simultaneous purification on Glutathione Sepharose™. The pGEX-6P series provides all three translational reading frames linked between the GST coding region and the multiple cloning site.

Collectively, the pGEX vectors provide all three translational reading frames beginning with the EcoR I restriction site. pGEX-1λT, pGEX-6P-1, pGEX-4T-1, and pGEX-5X-1 can directly accept and express cDNA inserts isolated from λ gt11 libraries.

Click on "ASCII" to download an unformatted sequence for use by a sequence analysis program. Click on "PDF" to download a formatted sequence and restriction site table. If you prefer accessing the sequence in *GenBank*, refer to the right-hand column for the GenBank accession number:

Vector	Unformatted	Formatted	GenBank Accession No.
pGEX-4T-1, 27-4580-01	*ASCII*	*PDF*	U13853
pGEX-4T-2, 27-4581-01	*ASCII*	*PDF*	U13854
pGEX-4T-3, 27-4583-01	*ASCII*	*PDF*	U13855
pGEX-5X-1, 27-4584-01	*ASCII*	*PDF*	U13856
pGEX-5X-2, 27-4585-01	*ASCII*	*PDF*	U13857
pGEX-5X-3, 27-4586-01	*ASCII*	*PDF*	U13858
pGEX-2TK, 27-4587-01	*ASCII*	*PDF*	U13851
pGEX-2T, 27-4801-01	*ASCII*	*PDF*	U13850
pGEX-3X, 27-4803-01	*ASCII*	*PDF*	U13852
pGEX-1 lambda T, 27-4805-01	*ASCII*	*PDF*	U13849
pGEX-6P-1, 27-4597-01	*ASCII*	*PDF*	U78872
pGEX-6P-2, 27-4598-01	*ASCII*	*PDF*	U78873
pGEX-6P-3, 27-4599-01	*ASCII*	*PDF*	U78874

Properties of pGEX Vectors • Induction: tac promoter inducible with 1-5 mM IPTG.

- **Expression:** Proteins are expressed as fusion proteins with the 26 kDa glutathione S-transferase (GST). The GST gene contains an ATG and ribosome-binding site, and is under control of

the **tac** promoter. A translation terminator is provided in each reading frame. The resulting fusion protein may be purified using the GST Purification Module (27-4570-01, -02; see GST Purification Modules.)

- **Enzymatic cleavage with PreScission™ Protease:** pGEX-6P-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with PreScission™ Protease. Because PreScission™ Protease has been engineered with a GST-tag, it can also be removed simultaneously with the GST portion of the fusion protein.
- **Enzymatic cleavage with thrombin:** pGEX-1 lambda T, pGEX-2T, pGEX-2TK, pGEX-4T-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with thrombin.
- **Enzymatic cleavage with factor Xa:** pGEX-3X, pGEX-5X-1, -2, -3 allow for removal of the GST carrier protein from the fusion protein by enzymatic cleavage with factor Xa.
- **Direct labeling in vitro:** pGEX-2TK allows for direct labeling of fusion proteins in vitro with 32P using the catalytic subunit of cAMP-dependent protein kinase.
- **Host(s):** E. coli. The plasmid provides lac Iq repressor.
- **Selectable marker(s):** Plasmid confers resistance to 100 µg/ml ampicillin.
- **Amplification:** Recommended.

• pGEX-2T Control Regions:

* Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
* MCS: 930-945
* Beta-lactamase gene region: Promoter: -10: 1309-1314; -35: 1286-1291; Start codon (ATG): 1356; Stop codon (TAA): 2214
* *lac*Iq gene region: Start codon (GTG): 3297; Stop codon (TGA): 4377
* Plasmid replication region: Site of replication initiation: 2974; Region necessary for replication: 2281-2977
* Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1020-998

• pGEX-2TK Control Regions:

* Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935;
* Coding for kinase recognition site: 936-950
* MCS: 951-966
* Beta-lactamase gene region: Promoter: -10: 1330-1335; -35: 1307-1312; Start codon (ATG): 1377; Stop codon (TAA): 2235
* *lac*Iq gene region: Start codon (GTG): 3318; Stop codon (TGA): 4398
* Plasmid replication region: Site of replication initiation: 2995; Region necessary for replication: 2302-2998

Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1041-1019

• pGEX-3X Control Regions:

* Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for Factor Xa cleavage: 921-932
* MCS: 934-949
* Beta-lactamase gene region: Promoter: -10: 1313-1318; -35: 1290-1295; Start codon (ATG): 1360; Stop codon (TAA): 2218
* *lac*Iq gene region: Start codon (GTG): 3301; Stop codon (TGA): 4381
* Plasmid replication region: Site of replication initiation: 2978; Region necessary for replication: 2285-2981
* Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1024-1002

• pGEX-1 Lambda T Control Regions:

* Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
* MCS: 930-944
* Beta-lactamase gene region: Promoter: -10: 1308-1313; -35: 1285-1290; Start codon (ATG): 1355; Stop codon (TAA): 2213
* *lac*Iq gene region: Start codon (GTG): 3296; Stop codon (TGA): 4376
* Plasmid replication region: Site of replication initiation: 2973; Region necessary for replication: 2280-2976
* Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1019-997

- **pGEX-4T-1 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
  * MCS: 930-966
  * Beta-lactamase gene region: Promoter: -10: 1330-1335; -35: 1307-1312; Start codon (ATG): 1377; Stop codon (TAA): 2235
  * *lac*Iq gene region: Start codon (GTG): 3318; Stop codon (TGA): 4398
  * Plasmid replication region: Site of replication initiation: 2995; Region necessary for replication: 2302-2998
  * Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1041-1019

- **pGEX-4T-2 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
  * MCS: 930-967
  * Beta-lactamase gene region: Promoter: -10: 1331-1336; -35: 1308-1313; Start codon (ATG): 1378; Stop codon (TAA): 2236
  * *lac*Iq gene region: Start codon (GTG): 3319; Stop codon (TGA): 4399
  * Plasmid replication region: Site of replication initiation: 2996; Region necessary for replication: 2303-2999
  * Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1042-1020

- **pGEX-4T-3 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for thrombin cleavage: 918-935
  * MCS: 930-965
  * Beta-lactamase gene region: Promoter: -10: 1329-1334; -35: 1306-1311; Start codon (ATG): 1376; Stop codon (TAA): 2234
  * *lac*Iq gene region: Start codon (GTG): 3317; Stop codon (TGA): 4397
  * Plasmid replication region: Site of replication initiation: 2994; Region necessary for replication: 2301-2997
  * Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1040-1018

- **pGEX-5X-1 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932
  * MCS: 934-969
  * Beta-lactamase gene region: Promoter: -10: 1333-1338; -35: 1310-1315; Start codon (ATG): 1380; Stop codon (TAA): 2238
  * *lac*Iq gene region: Start codon (GTG): 3321; Stop codon (TGA): 4401
  * Plasmid replication region: Site of replication initiation: 2998; Region necessary for replication: 2305-3001
  * Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1044-1022

- **pGEX-5X-2 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932
  * MCS: 934-970
  * Beta-lactamase gene region: Promoter: -10: 1334-1339; -35: 1311-1316; Start codon (ATG): 1381; Stop codon (TAA): 2239
  * *lac*Iq gene region: Start codon (GTG): 3322; Stop codon (TGA): 4402
  * Plasmid replication region: Site of replication initiation: 2999; Region necessary for replication: 2306-3002
  * Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1045-1023

- **pGEX-5X-3 Control Regions:**
  * Glutathione S-transferase gene region: *tac* promoter: -10: 205-211; -35: 183-188; *lac* operator: 217-237; Ribosome binding site for GST: 244; Start codon (ATG) for GST: 258; Coding region for factor Xa cleavage: 921-932

* MCS: 934-971
* Beta-lactamase gene region: Promoter: -10: 1335-1340; -35: 1312-1317; Start codon (ATG): 1382; Stop codon (TAA): 2240
* *lacIq* gene region: Start codon (GTG): 3323; Stop codon (TGA): 4403
* Plasmid replication region: Site of replication initiation: 3000; Region necessary for replication: 2307-3003
* Sequencing primers: 5' pGEX Sequencing Primer binds nucleotides 869-891; 3' pGEX Sequencing Primer binds nucleotides 1046-1024

References

1. Kaelin, W.G. *et al., Cell* ***70,*** 351 (1992).

1: Science. 1996 Sep 6;273(5280):1392-5. Related Articles, Links

Comment in:
- Science. 1996 Sep 6;273(5280):1336.

Crystal structure of the Aequorea victoria green fluorescent protein.

Ormo M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ.

Institute of Molecular Biology and Department of Physics, University of Oregon, Eugene, OR 97403-1226, USA.

The green fluorescent protein (GFP) from the Pacific Northwest jellyfish Aequorea victoria has generated intense interest as a marker for gene expression and localization of gene products. The chromophore, resulting from the spontaneous cyclization and oxidation of the sequence -Ser65 (or Thr65)-Tyr66-Gly67-, requires the native protein fold for both formation and fluorescence emission. The structure of Thr65 GFP has been determined at 1.9 angstrom resolution. The protein fold consists of an 11-stranded beta barrel with a coaxial helix, with the chromophore forming from the central helix. Directed mutagenesis of one residue adjacent to the chromophore, Thr203, to Tyr or His results in significantly red-shifted excitation and emission maxima.

PMID: 8703075 [PubMed - indexed for MEDLINE]

1: EMBO J. 1993 May;12(5):1767-74.

Crystal structure of a flavoprotein related to the subunits of bacterial luciferase.

Moore SA, James MN, O'Kane DJ, Lee J.

Department of Biochemistry, University of Alberta, Edmonton, Canada.

The molecular structure of the luxF protein from the bioluminescent bacterium Photobacterium leiognathi has been determined by X-ray diffraction techniques and refined to a conventional R-factor of 17.8% at 2.3 A resolution. The 228 amino acid polypeptide exists as a symmetrical homodimer and 33% of the monomer's solvent-accessible surface area is buried upon dimerization. The monomer displays a novel fold that contains a central seven-stranded beta-barrel. The solvent-exposed surface of the monomer is covered by seven alpha-helices, whereas the dimer interface is primarily a flat surface composed of beta-strands. The protein monomer binds two molecules of flavin mononucleotide, each of which has C6 of the flavin isoalloxazine moiety covalently attached to the C3' carbon atom of myristic acid. Both myristyl groups of these adducts are buried within the hydrophobic core of the protein. One of the cofactors contributes to interactions at the dimer interface. The luxF protein displays considerable amino acid sequence homology with both alpha- and beta-subunits of bacterial luciferase, especially the beta-subunit. Conserved amino acid residues shared between luxF and the luciferase subunits cluster predominantly in two distinct regions of the luxF protein molecule. These homologous regions in the luciferase subunits probably share a three-dimensional fold similar to that of the luxF protein.

PMID: 8491169 [PubMed - indexed for MEDLINE]

1: Nat Biotechnol. 1996 Oct;14(10):1246-51. Related Articles, Links

nature biotechnology

The molecular structure of green fluorescent protein.

Yang F, Moss LG, Phillips GN Jr.

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005-1892, USA.

The crystal structure of recombinant wild-type green fluorescent protein (GFP) has been solved to a resolution of 1.9 A by multiwavelength anomalous dispersion phasing methods. The protein is in the shape of a cylinder, comprising 11 strands of beta-sheet with an alpha-helix inside and short helical segments on the ends of the cylinder. This motif, with beta-structure on the outside and alpha-helix on the inside, represents a new protein fold, which we have named the beta-can. Two protomers pack closely together to form a dimer in the crystal. The fluorophores are protected inside the cylinders, and their structures are consistent with the formation of aromatic systems made up of Tyr66 with reduction of its C alpha-C beta bond coupled with cyclization of the neighboring glycine and serine residues. The environment inside the cylinder explains the effects of many existing mutants of GFP and suggests specific side chains that could be modified to change the spectral properties of GFP. Furthermore, the identification of the dimer contacts may allow mutagenic control of the state of assembly of the protein.

PMID: 9631087 [PubMed - indexed for MEDLINE]

1: Biochemistry. 1997 Aug 12;36(32):9759-65.

Crystal structure and photodynamic behavior of the blue emission variant Y66H/Y145F of green fluorescent protein.

Wachter RM, King BA, Heim R, Kallio K, Tsien RY, Boxer SG, Remington SJ.

Institute of Molecular Biology, Department of Physics, University of Oregon, Eugene 97403, USA.

The crystal structure of a blue emission variant (Y66H/Y145F) of the Aequorea victoria green fluorescent protein has been determined by molecular replacement and the model refined. The crystallographic R-factor is 18.1% for all data from 20 to 2.1 A, and the model geometry is excellent. The chromophore is non-native and is autocatalytically generated from the internal tripeptide Ser65-His66-Gly67. The final electron density maps indicate that the formation of the chromophore is complete, including 1,2 dehydration of His66 as indicated by the planarity of the chromophore. The chromophore is in the cis conformation, with no evidence for any substantial fraction of the trans configuration or uncyclized apoprotein, and is well-shielded from bulk solvent by the folded protein. These characteristics indicate that the machinery for production of the chromophore from a buried tripeptide unit is not only intact but also highly efficient in spite of a major change in chromophore chemical structure. Nevertheless, there are significant rearrangements in the hydrogen bond configuration around the chromophore as compared to wild-type, indicating flexibility of the active site. pH titration of the intact protein and the chromopeptide (pKa1 = 4.9 +/- 0.1, pKa2 = 12.0 +/- 0.1) suggests that the predominant form of the chromophore in the intact protein is electrically neutral. In contrast to the wild-type protein [Chattoraj, M., King, B. A., Bublitz, G. U., & Boxer, S. G. (1996) Proc. Natl. Acad. Sci. U.S.A., 8362-8367], femtosecond fluorescence up-conversion spectroscopy of the intact protein and a partially deuterated form strongly suggests that excited-state proton transfer is not coupled to fluorescence emission.

PMID: 9245407 [PubMed - indexed for MEDLINE]

1: Proc Natl Acad Sci U S A. 1997 Mar 18;94(6):2306-11.

Structural basis for dual excitation and photoisomerization of the Aequorea victoria green fluorescent protein.

Brejc K, Sixma TK, Kitts PA, Kain SR, Tsien RY, Ormo M, Remington SJ.

Netherlands Cancer Institute, Department of Molecular Carcinogenesis, Amsterdam.

The 2.1-A resolution crystal structure of wild-type green fluorescent protein and comparison of it with the recently determined structure of the Ser-65 --> Thr (S65T) mutant explains the dual wavelength absorption and photoisomerization properties of the wild-type protein. The two absorption maxima are caused by a change in the ionization state of the chromophore. The equilibrium between these states appears to be governed by a hydrogen bond network that permits proton transfer between the chromophore and neighboring side chains. The predominant neutral form of the fluorophore maximally absorbs at 395 nm. It is maintained by the carboxylate of Glu-222 through electrostatic repulsion and hydrogen bonding via a bound water molecule and Ser-205. The ionized form of the fluorophore, absorbing at 475 nm, is present in a minor fraction of the native protein. Glu-222 donates its charge to the fluorophore by proton abstraction through a hydrogen bond network, involving Ser-205 and bound water. Further stabilization of the ionized state of the fluorophore occurs through a rearrangement of the side chains of Thr-203 and His-148. UV irradiation shifts the ratio of the two absorption maxima by pumping a proton relay from the neutral chromophore's excited state to Glu-222. Loss of the Ser-205-Glu-222 hydrogen bond and isomerization of neutral Glu-222 explains the slow return to the equilibrium dark-adapted state of the chromophore. In the S65T structure, steric hindrance by the extra methyl group stabilizes a hydrogen bonding network, which prevents ionization of Glu-222. Therefore the fluorophore is permanently ionized, causing only a 489-nm excitation peak. This new understanding of proton redistribution in green fluorescent protein should enable engineering of environmentally sensitive fluorescent indicators and UV-triggered fluorescent markers of protein diffusion and trafficking in living cells.

1: EMBO J. 1993 May;12(5):1767-74.

Crystal structure of a flavoprotein related to the subunits of bacterial luciferase.

Moore SA, James MN, O'Kane DJ, Lee J.

Department of Biochemistry, University of Alberta, Edmonton, Canada.

The molecular structure of the luxF protein from the bioluminescent bacterium Photobacterium leiognathi has been determined by X-ray diffraction techniques and refined to a conventional R-factor of 17.8% at 2.3 A resolution. The 228 amino acid polypeptide exists as a symmetrical homodimer and 33% of the monomer's solvent-accessible surface area is buried upon dimerization. The monomer displays a novel fold that contains a central seven-stranded beta-barrel. The solvent-exposed surface of the monomer is covered by seven alpha-helices, whereas the dimer interface is primarily a flat surface composed of beta-strands. The protein monomer binds two molecules of flavin mononucleotide, each of which has C6 of the flavin isoalloxazine moiety covalently attached to the C3' carbon atom of myristic acid. Both myristyl groups of these adducts are buried within the hydrophobic core of the protein. One of the cofactors contributes to interactions at the dimer interface. The luxF protein displays considerable amino acid sequence homology with both alpha- and beta-subunits of bacterial luciferase, especially the beta-subunit. Conserved amino acid residues shared between luxF and the luciferase subunits cluster predominantly in two distinct regions of the luxF protein molecule. These homologous regions in the luciferase subunits probably share a three-dimensional fold similar to that of the luxF protein.

PMID: 8491169 [PubMed - indexed for MEDLINE]

1: Biochemistry. 1995 May 23;34(20):6581-6. Related Articles, Links

Three-dimensional structure of bacterial luciferase from Vibrio harveyi at 2.4 A resolution.

Fisher AJ, Raushel FM, Baldwin TO, Rayment I.

Department of Biochemistry, University of Wisconsin, Madison 53705, USA.

Luciferases are a class of enzymes that generate light in the visible spectrum. Luciferase from luminous marine bacteria is an alpha-beta heterodimer monooxygenase that catalyzes the oxidation of FMNH2 and a long-chain aliphatic aldehyde. The X-ray crystal structure of bacterial luciferase from Vibrio harveyi has been determined to 2.4 A resolution. The structure was solved by a combination of multiple isomorphous replacement and molecular averaging between the two heterodimers in the asymmetric unit. Each subunit folds into a (beta/alpha)8 barrel motif, and dimerization is mediated through a parallel four-helix bundle centered on a pseudo 2-fold axis that relates the structurally similar subunits. The vicinity of the active site has been identified on the alpha subunit by correlations with similar protein motifs and previous biochemical studies. The structure presented here represents the first molecular model of a bioluminescent enzyme.

PMID: 7756289 [PubMed - indexed for MEDLINE]

1: Biochemistry. 1996 Oct 22;35(42):13531-9. Related Articles, Links

Flavin reductase P: structure of a dimeric enzyme that reduces flavin.

Tanner JJ, Lei B, Tu SC, Krause KL.

Department of Biochemical and Biophysical Sciences, University of Houston, Texas 77204-5934, USA.

We report the structure of an NADPH:FMN oxidoreductase (flavin reductase P) that is involved in bioluminescence by providing reduced FMN to luciferase. The 1.8 A crystal structure of flavin reductase P from Vibrio harveyi was solved by multiple isomorphous replacement and reveals that the enzyme is a unique dimer of interlocking subunits, with 9352 A2 of surface area buried in the dimer interface. Each subunit comprises two domains. The first domain consists of a four-stranded antiparallel beta-sheet flanked by helices on either side. The second domain reaches out from one subunit and embraces the other subunit and is responsible for interlocking the two subunits. Our structure explains why flavin reductase P is specific for FMN as cofactor. FMN is recognized and tightly bound by a network of 16 hydrogen bonds, while steric considerations prevent the binding of FAD. A flexible loop containing a Lys and an Arg could account for the NADPH specificity. The structure reveals information about several aspects of the catalytic mechanism. For example, we show that the first step in catalysis, which is hydride transfer from C4 of NADPH to cofactor FMN, involves addition to the re face of the FMN, probably at the N5 position. The limited accessibility of the FMN binding pocket and the extensive FMN-protein hydrogen bond network are consistent with the observed ping-pong bisubstrate--biproduct reaction kinetics. Finally, we propose a model for how flavin reductase P might shuttle electrons between NADPH and luciferase.

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1: Protein Sci. 1997 Jan;6(1):13-23.

Structure of the beta 2 homodimer of bacterial luciferase from Vibrio harveyi: X-ray analysis of a kinetic protein folding trap.

Thoden JB, Holden HM, Fisher AJ, Sinclair JF, Wesenberg G, Baldwin TO, Rayment I.

Institute for Enzyme Research, University of Wisconsin, Madison 53705, USA.

Luciferase, as isolated from Vibrio harveyi, is an alpha beta heterodimer. When allowed to fold in the absence of the alpha subunit, either in vitro or in vivo, the beta subunit of enzyme will form a kinetically stable homodimer that does not unfold even after prolonged incubation in 5 M urea at pH 7.0 and 18 degrees C. This form of the beta subunit, arising via kinetic partitioning on the folding pathway, appears to constitute a kinetically trapped alternative to the heterodimeric enzyme (Sinclair JF, Ziegler MM, Baldwin TO. 1994. Kinetic partitioning during protein folding yields multiple native states. Nature Struct Biol 1: 320-326). Here we describe the X-ray crystal structure of the beta 2 homodimer of luciferase from V. harveyi determined and refined at 1.95 A resolution. Crystals employed in the investigational belonged to the orthorhombic space group P2(1)2(1)2(1) with unit cell dimensions of a = 58.8 A, b = 62.0 A, and c = 218.2 A and contained one dimer per asymmetric unit. Like that observed in the functional luciferase alpha beta heterodimer, the major tertiary structural motif of each beta subunit consists of an (alpha/beta)8 barrel (Fisher AJ, Raushel FM, Baldwin TO, Rayment I. 1995. Three-dimensional structure of bacterial luciferase from Vibrio harveyi at 2.4 A resolution. Biochemistry 34: 6581-6586). The root-mean-square deviation of the alpha-carbon coordinates between the beta subunits of the hetero- and homodimers is 0.7 A. This high resolution X-ray analysis demonstrated that "domain" or "loop" swapping has not occurred upon formation of the beta 2 homodimer and thus the stability of the beta 2 species to denaturation cannot be explained in such simple terms. In fact, the subunit:subunit interfaces observed in both the beta 2 homodimer and alpha beta heterodimer are remarkably similar in hydrogen-bonding patterns and buried surface areas.

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1: Biochemistry. 1997 Jan 28;36(4):665-72. Related Articles, Links

Structure of bacterial luciferase beta 2 homodimer: implications for flavin binding.

Tanner JJ, Miller MD, Wilson KS, Tu SC, Krause KL.

Department of Biochemical and Biophysical Sciences, University of Houston, Texas 77204-5934, USA.

The crystal structure of the beta 2 homodimer of Vibrio harveyi luciferase has been determined to 2.5 A resolution by molecular replacement. Crystals were grown serendipitously using the alpha beta form of the enzyme. The subunits of the homodimer share considerable structural homology to the beta subunit of the alpha beta luciferase heterodimer. The four C-terminal residues that are disordered in the alpha beta structure are fully resolved in our structure. Four peptide bonds have been flipped relative to their orientations in the beta subunit of the alpha beta structure. The dimer interface of the homodimer is smaller than the interface of the heterodimer in terms of buried surface area and number of hydrogen bonds and salt links. Inspection of the subunits of our structure suggests that FMNH2 cannot bind to the beta 2 enzyme at the site that has been proposed for the alpha beta enzyme. However, we do uncover a potential FMNH2 binding pocket in the dimer interface, and we model FMN into this site. This proposed flavin binding motif is consistent with several lines of biochemical and structural evidence and leads to several conclusions. First, only one FMNH2 binds per homodimer. Second, we predict that reduced FAD and riboflavin should be poor substrates for beta 2. Third, the reduced activity of beta 2 compared to alpha beta is due to solvent exposure of the isoalloxazine ring in the beta 2 active site. Finally, we raise the question of whether our proposed flavin binding site could also be the binding site for flavin in the alpha beta enzyme.

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